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(54) **HYBRID HEPATOCYTE GROWTH FACTOR GENE HAVING HIGH EXPRESSION EFFICIENCY OF TWO HETEROTYPES OF HEPATOCYTE GROWTH FACTOR**

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This patent is subject to a terminal disclaimer.

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(30) **Foreign Application Priority Data**

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(51) **Int. Cl.**

C12P 21/06	(2006.01)
C12P 21/04	(2006.01)
C12N 15/09	(2006.01)

(52) **U.S. Cl.** **435/69.1**; 435/69.4; 435/70.1; 435/71.1

(58) **Field of Classification Search** .... None
See application file for complete search history.

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(57) **ABSTRACT**

The present invention relates to a hybrid Hepatocyte Growth Factor (HGF) gene which is prepared by inserting an inherent or foreign intron between exons 4 and 5 in HGF cDNA, which has a base sequence of SEQ ID NO: 2. The gene has high expression efficiency and simultaneously expresses two heterotypes of HGF and dHGF (deleted variant HGF). Further the gene may be used for treating or preventing ischemic or liver diseases.

21 Claims, 12 Drawing Sheets

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FIG. 1

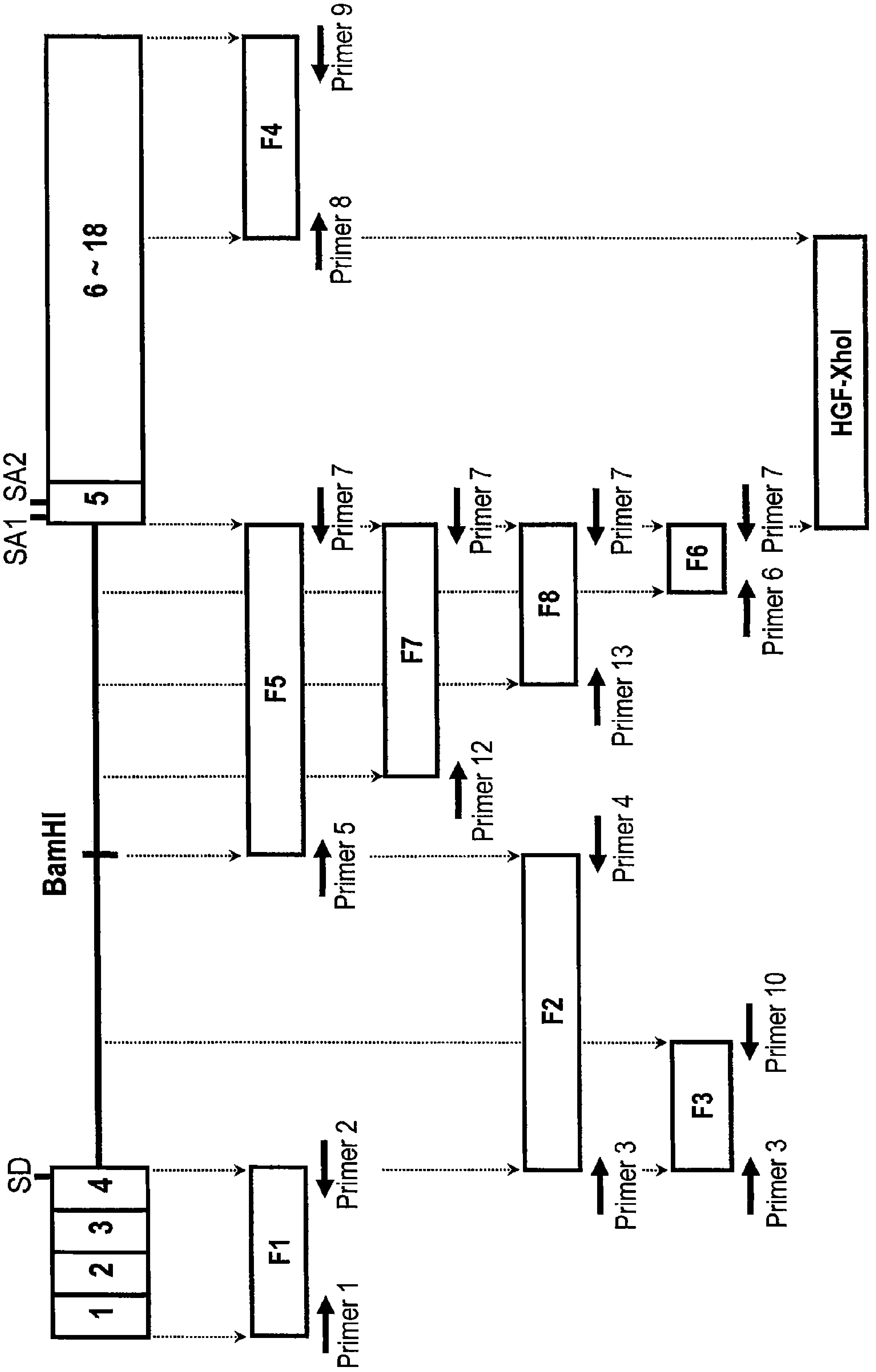

SD
1
2
3
4
BamHI
SA1
SA2
5
6 ~ 18
F1
F2
F3
F4
F5
F6
F7
F8
HGF-XhoI
Primer 1
Primer 2
Primer 3
Primer 3
Primer 10
Primer 4
Primer 5
Primer 12
Primer 13
Primer 6
Primer 7
Primer 7
Primer 7
Primer 7
Primer 8
Primer 9

FIG. 2

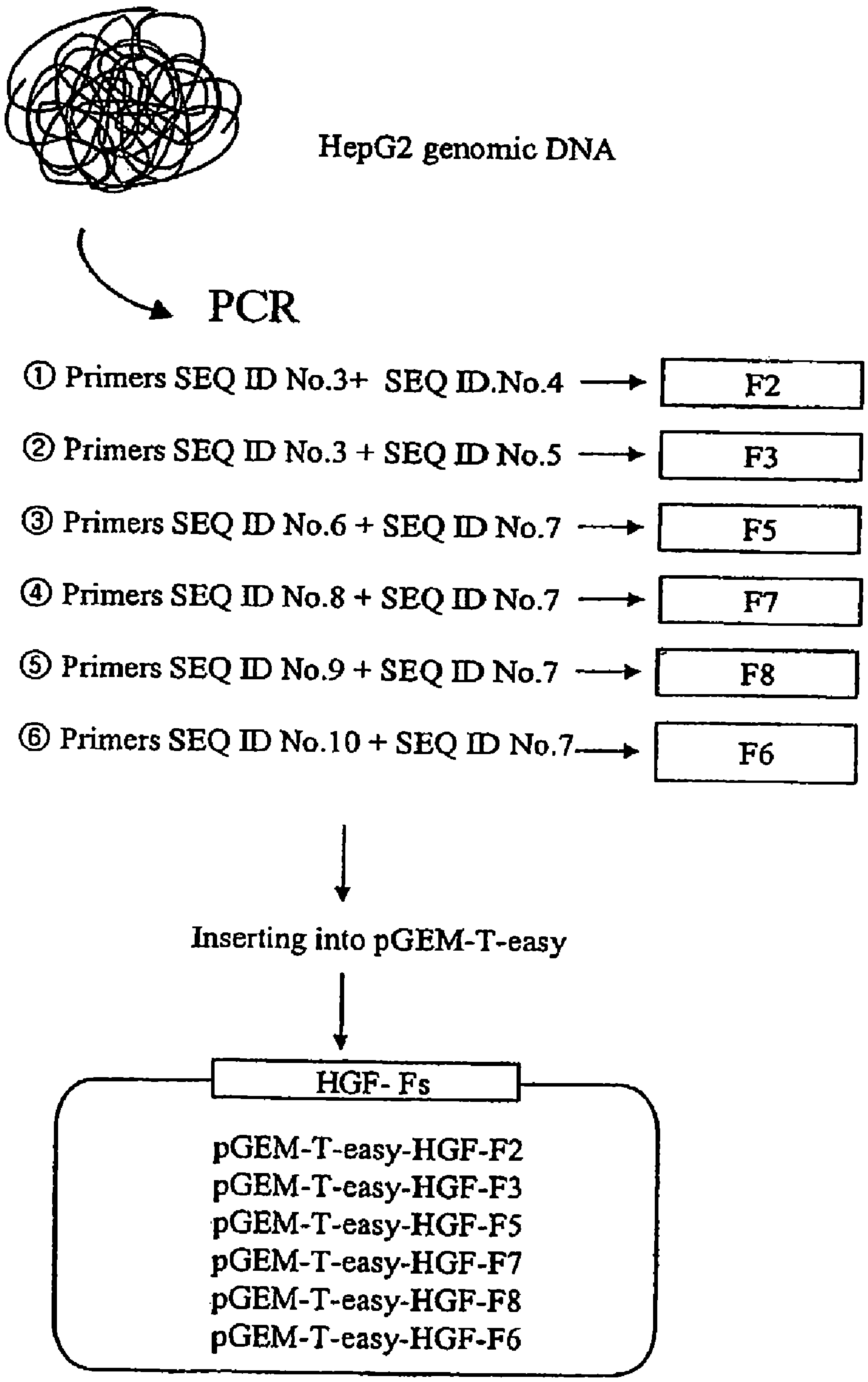

HepG2 genomic DNA
PCR
① Primers SEQ ID No.3+ SEQ ID.No.4
F2
② Primers SEQ ID No.3 + SEQ ID No.5
F3
③ Primers SEQ ID No.6 + SEQ ID No.7
F5
④ Primers SEQ ID No.8 + SEQ ID No.7
F7
⑤ Primers SEQ ID No.9 + SEQ ID No.7
F8
⑥ Primers SEQ ID No.10 + SEQ ID No.7
F6
Inserting into pGEM-T-easy
HGF- Fs
pGEM-T-easy-HGF-F2
pGEM-T-easy-HGF-F3
pGEM-T-easy-HGF-F5
pGEM-T-easy-HGF-F7
pGEM-T-easy-HGF-F8
pGEM-T-easy-HGF-F6

FIG. 3

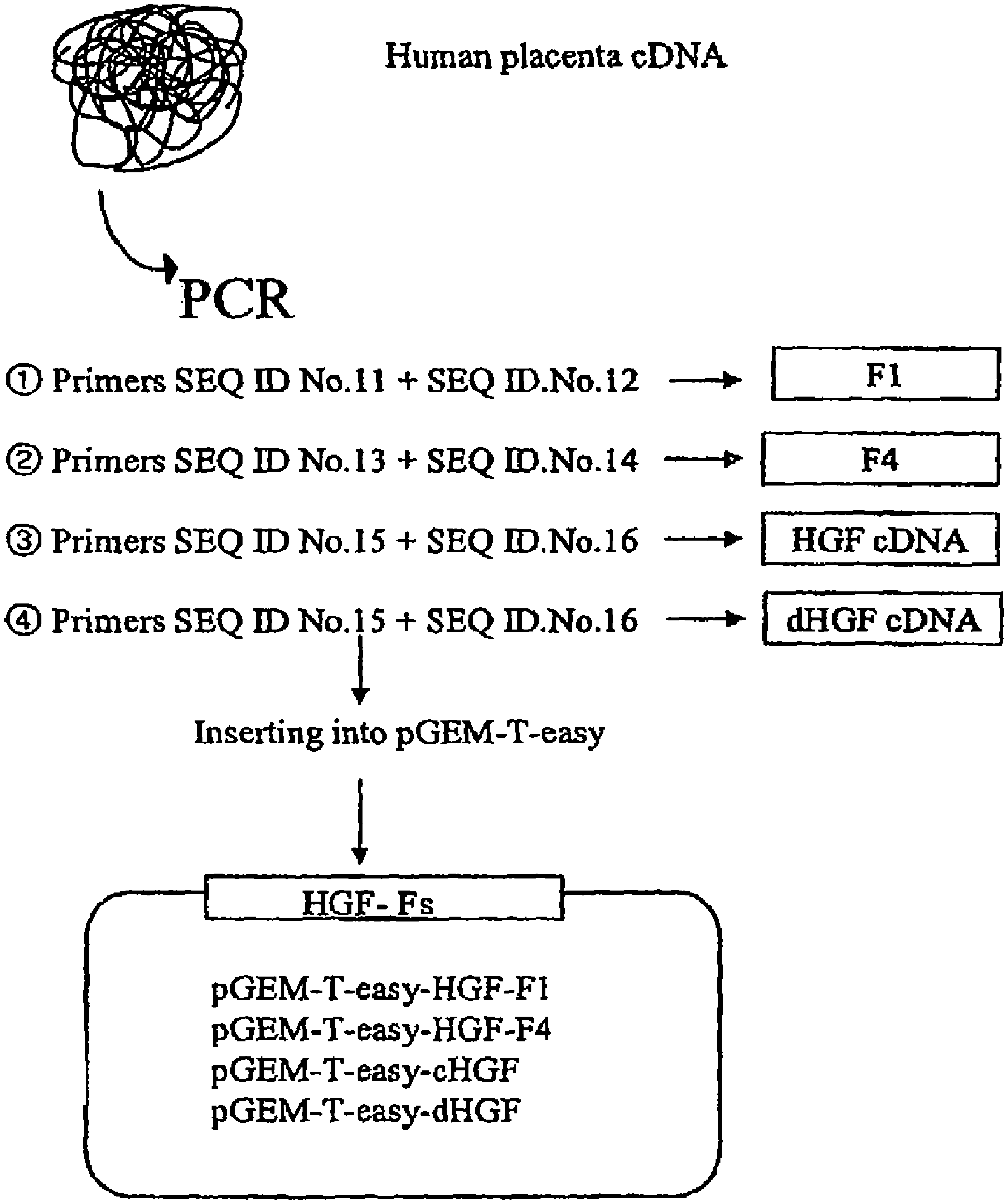

Human placenta cDNA
PCR
① Primers SEQ ID No.11 + SEQ ID.No.12
F1
② Primers SEQ ID No.13 + SEQ ID.No.14
F4
③ Primers SEQ ID No.15 + SEQ ID.No.16
HGF cDNA
④ Primers SEQ ID No.15 + SEQ ID.No.16
dHGF cDNA
Inserting into pGEM-T-easy
HGF- Fs
pGEM-T-easy-HGF-F1
pGEM-T-easy-HGF-F4
pGEM-T-easy-cHGF
pGEM-T-easy-dHGF

FIG. 4A

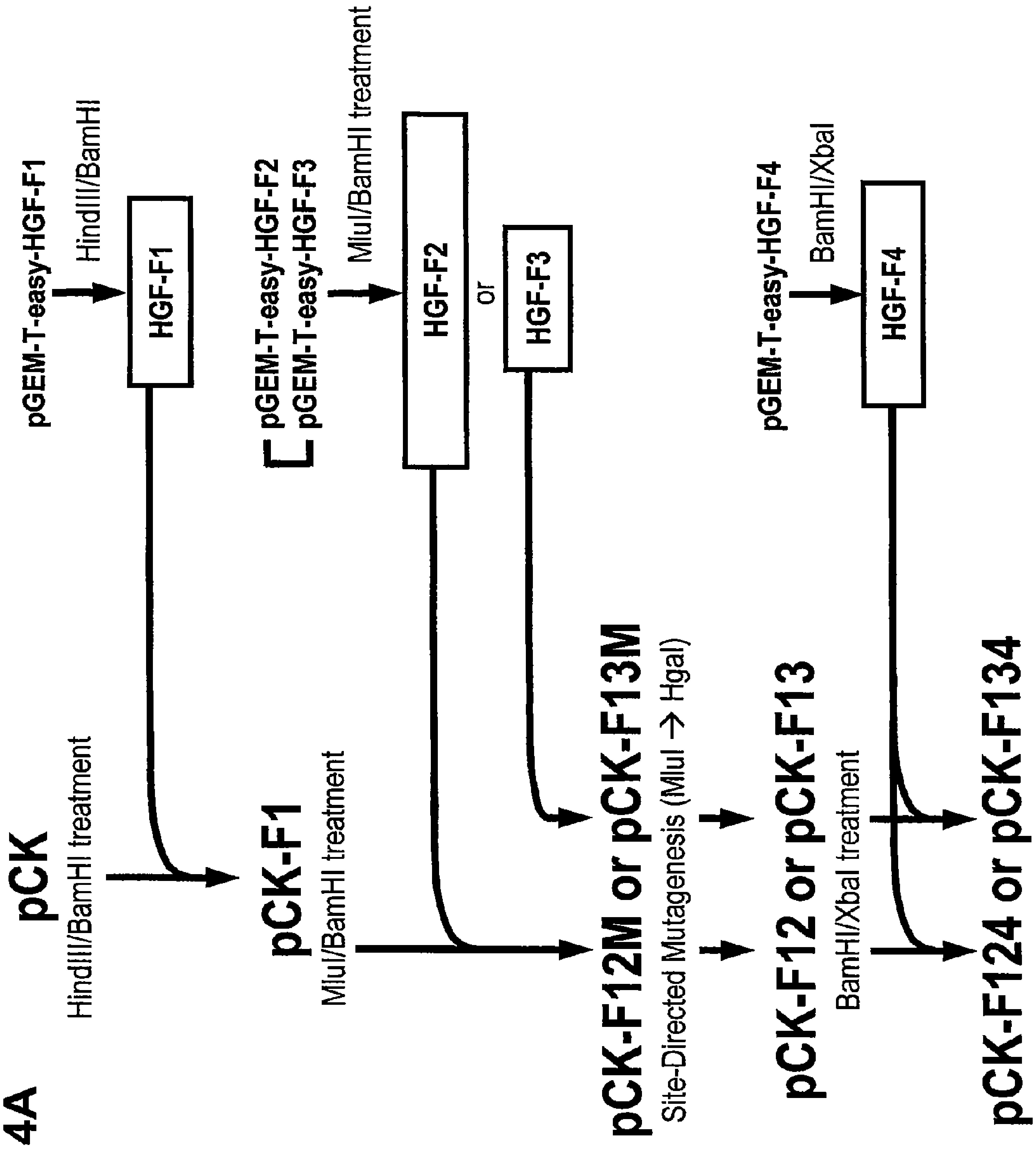

pCK
HindIII/BamHI treatment
pGEM-T-easy-HGF-F1
HindIII/BamHI
HGF-F1
pCK-F1
MluI/BamHI treatment
pGEM-T-easy-HGF-F2
pGEM-T-easy-HGF-F3
MluI/BamHI treatment
HGF-F2
or
HGF-F3
pCK-F12M or pCK-F13M
Site-Directed Mutagenesis (MluI → HgaI)
pCK-F12 or pCK-F13
pGEM-T-easy-HGF-F4
BamHI/XbaI
HGF-F4
BamHI/XbaI treatment
pCK-F124 or pCK-F134

FIG. 4B

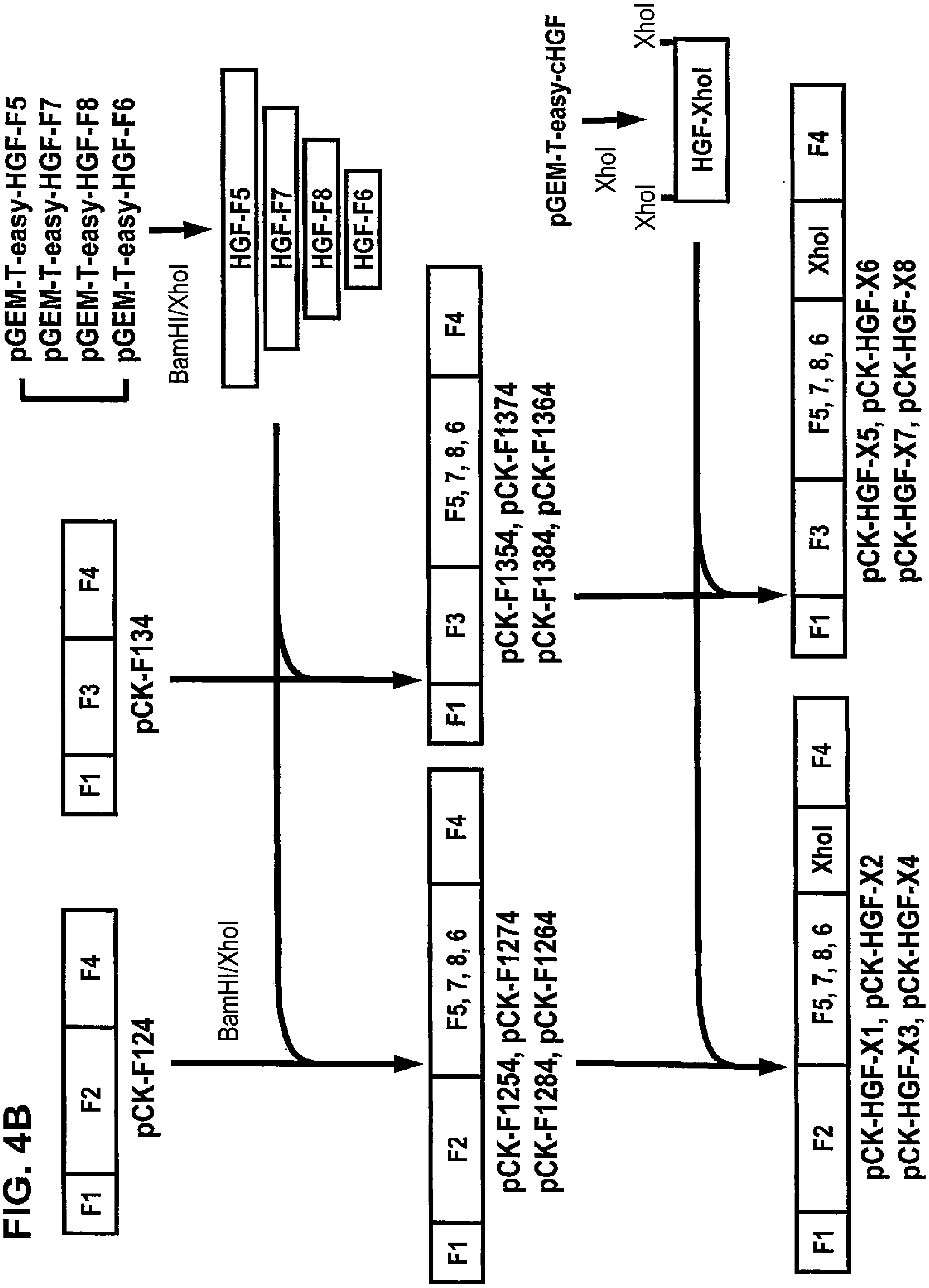

F1
F2
F4
pCK-F124
F1
F3
F4
pCK-F134
pGEM-T-easy-HGF-F5
pGEM-T-easy-HGF-F7
pGEM-T-easy-HGF-F8
pGEM-T-easy-HGF-F6
BamHI/XhoI
HGF-F5
HGF-F7
HGF-F8
HGF-F6
BamHI/XhoI
F1
F2
F5, 7, 8, 6
F4
pCK-F1254, pCK-F1274
pCK-F1284, pCK-F1264
F1
F3
F5, 7, 8, 6
F4
pCK-F1354, pCK-F1374
pCK-F1384, pCK-F1364
pGEM-T-easy-cHGF
XhoI
XhoI
HGF-XhoI
XhoI
F1
F2
F5, 7, 8, 6
XhoI
F4
pCK-HGF-X1, pCK-HGF-X2
pCK-HGF-X3, pCK-HGF-X4
F1
F3
F5, 7, 8, 6
XhoI
F4
pCK-HGF-X5, pCK-HGF-X6
pCK-HGF-X7, pCK-HGF-X8

FIG. 5

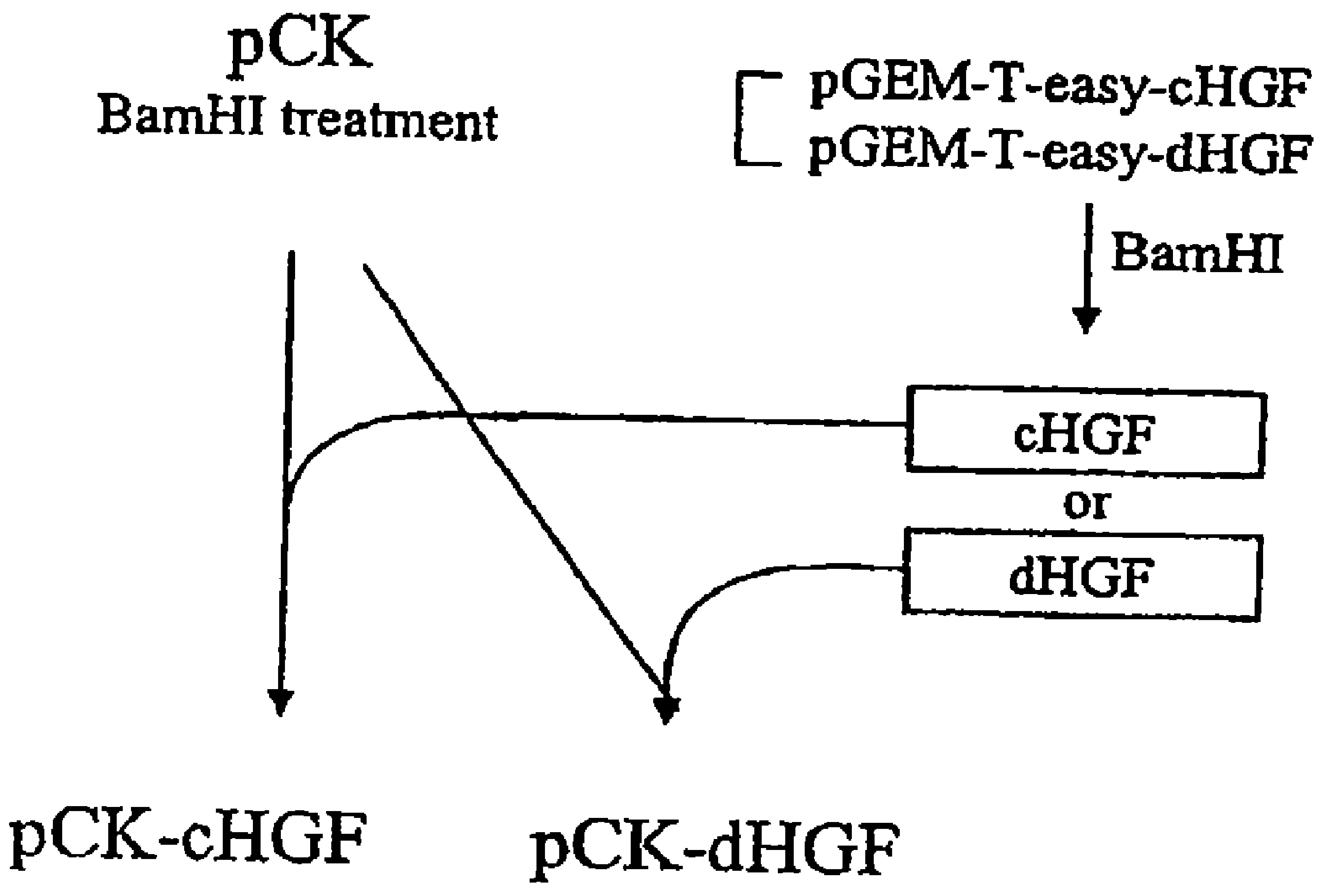

pCK
BamHI treatment
pGEM-T-easy-cHGF
pGEM-T-easy-dHGF
BamHI
cHGF
or
dHGF
pCK-cHGF
pCK-dHGF

FIG. 6

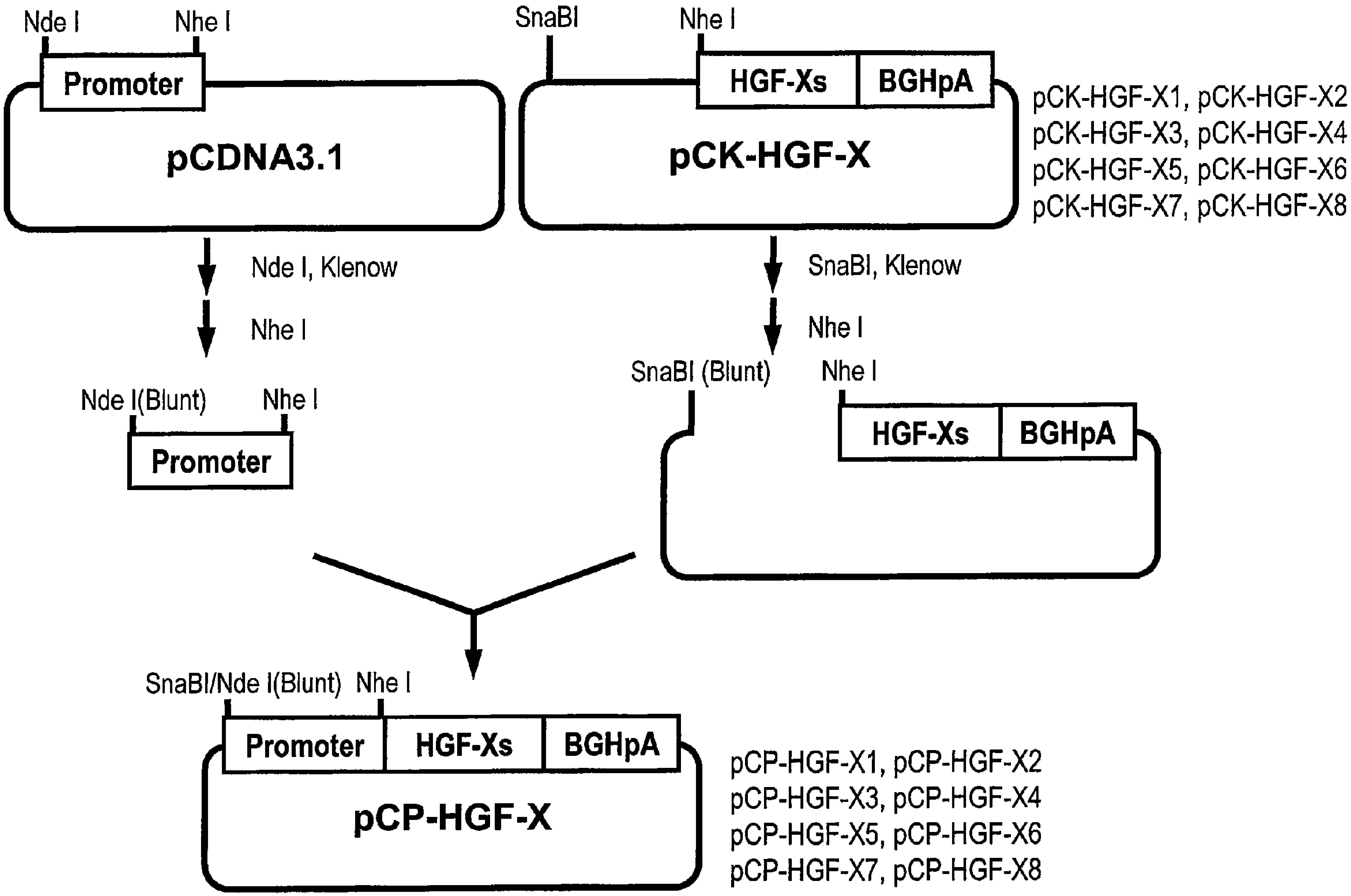

Nde I
Nhe I
Promoter
pCDNA3.1
SnaBI
Nhe I
HGF-Xs
BGHpA
pCK-HGF-X
pCK-HGF-X1, pCK-HGF-X2
pCK-HGF-X3, pCK-HGF-X4
pCK-HGF-X5, pCK-HGF-X6
pCK-HGF-X7, pCK-HGF-X8
Nde I, Klenow
Nhe I
SnaBI, Klenow
Nhe I
Nde I(Blunt)
Nhe I
Promoter
SnaBI (Blunt)
Nhe I
HGF-Xs
BGHpA
SnaBI/Nde I(Blunt)
Nhe I
Promoter
HGF-Xs
BGHpA
pCP-HGF-X
pCP-HGF-X1, pCP-HGF-X2
pCP-HGF-X3, pCP-HGF-X4
pCP-HGF-X5, pCP-HGF-X6
pCP-HGF-X7, pCP-HGF-X8

FIG. 7

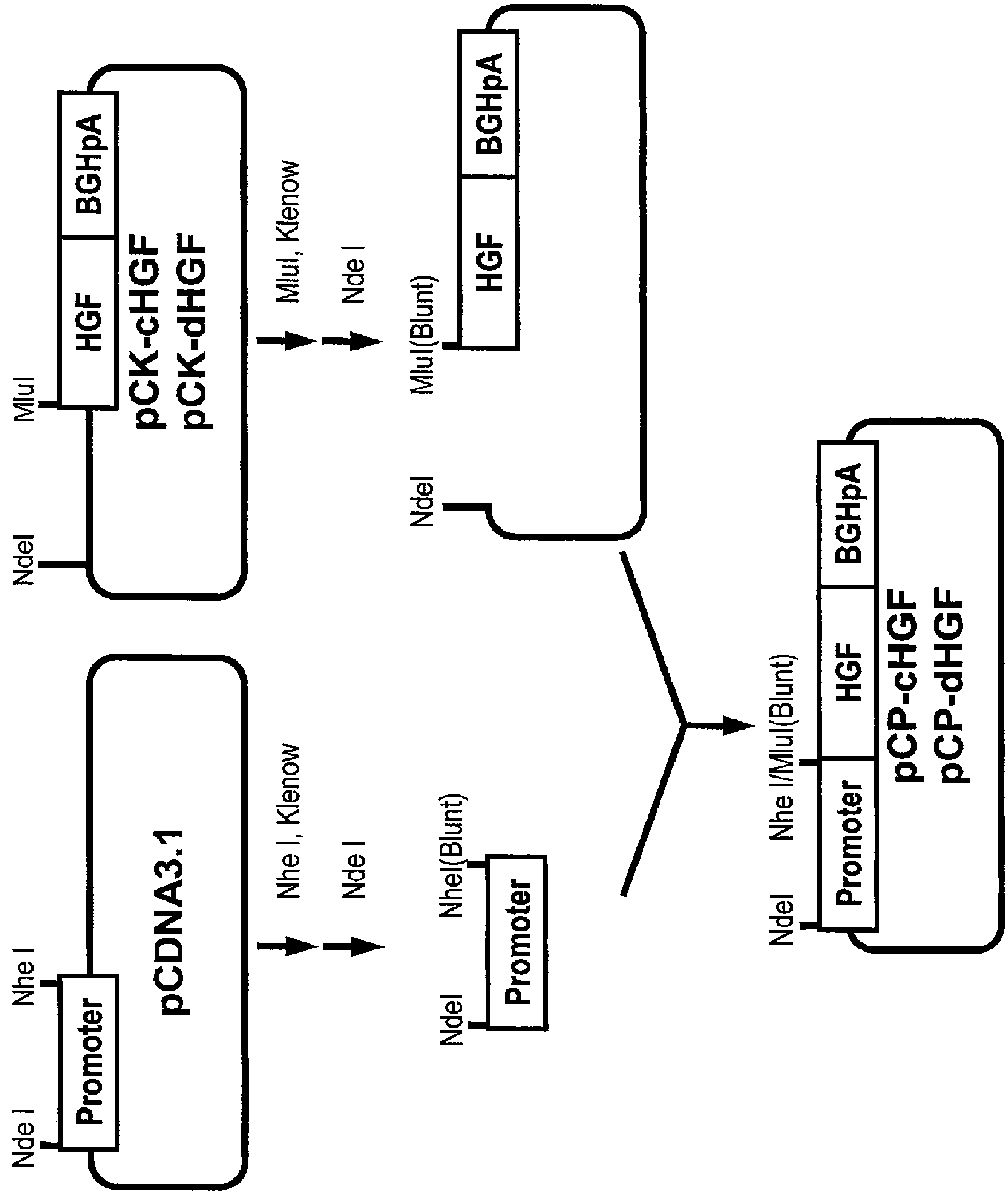

Nde I
Nhe I
Promoter
pCDNA3.1
Nhe I, Klenow
Nde I
NdeI
NheI(Blunt)
Promoter
NdeI
MluI
HGF
BGHpA
pCK-cHGF
pCK-dHGF
MluI, Klenow
Nde I
NdeI
MluI(Blunt)
HGF
BGHpA
NdeI
Nhe I/MluI(Blunt)
Promoter
HGF
BGHpA
pCP-cHGF
pCP-dHGF

FIG. 8

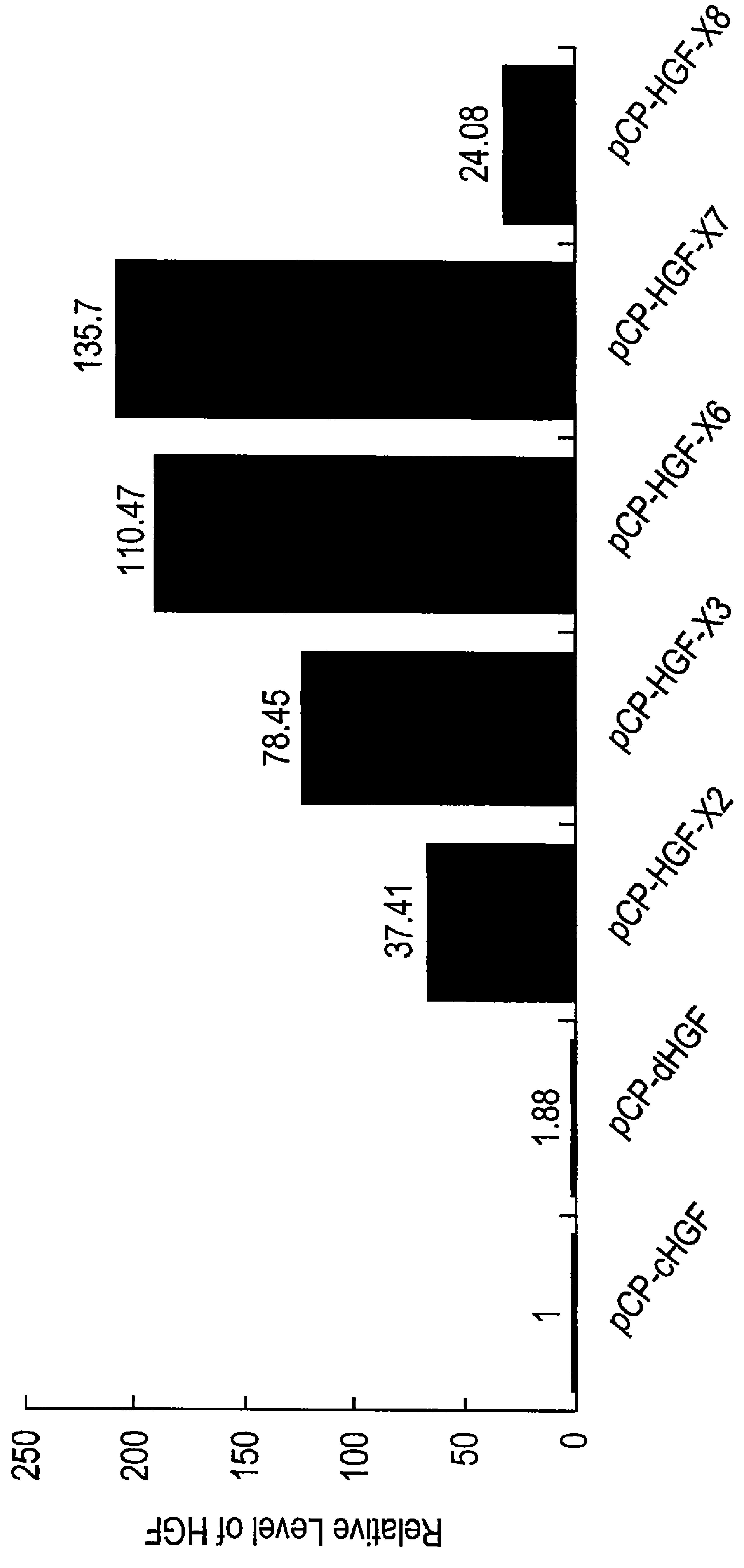

Relative Level of HGF
0
50
100
150
200
250
1
1.88
37.41
78.45
110.47
135.7
24.08
pCP-cHGF
pCP-dHGF
pCP-HGF-X2
pCP-HGF-X3
pCP-HGF-X6
pCP-HGF-X7
pCP-HGF-X8

FIG. 9

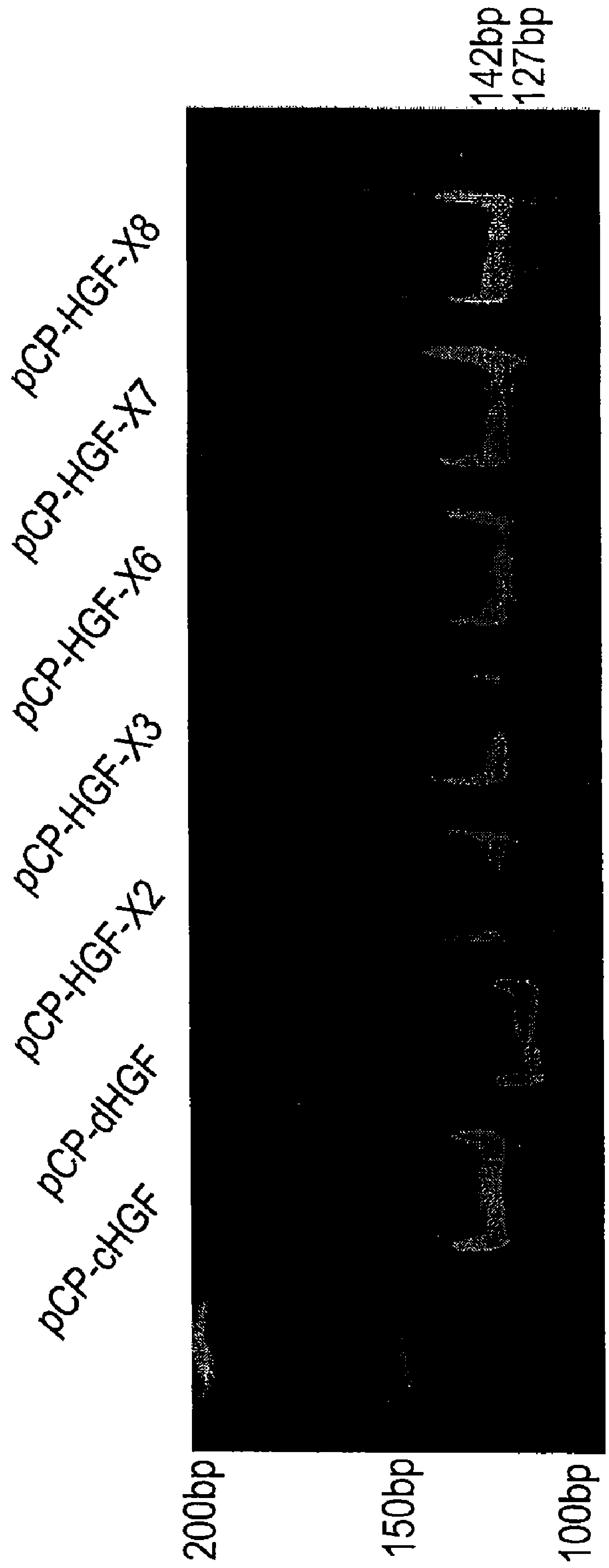

pCP-HGF-X8
pCP-HGF-X7
pCP-HGF-X6
pCP-HGF-X3
pCP-HGF-X2
pCP-dHGF
pCP-cHGF
142bp
127bp
200bp
150bp
100bp

FIG. 10

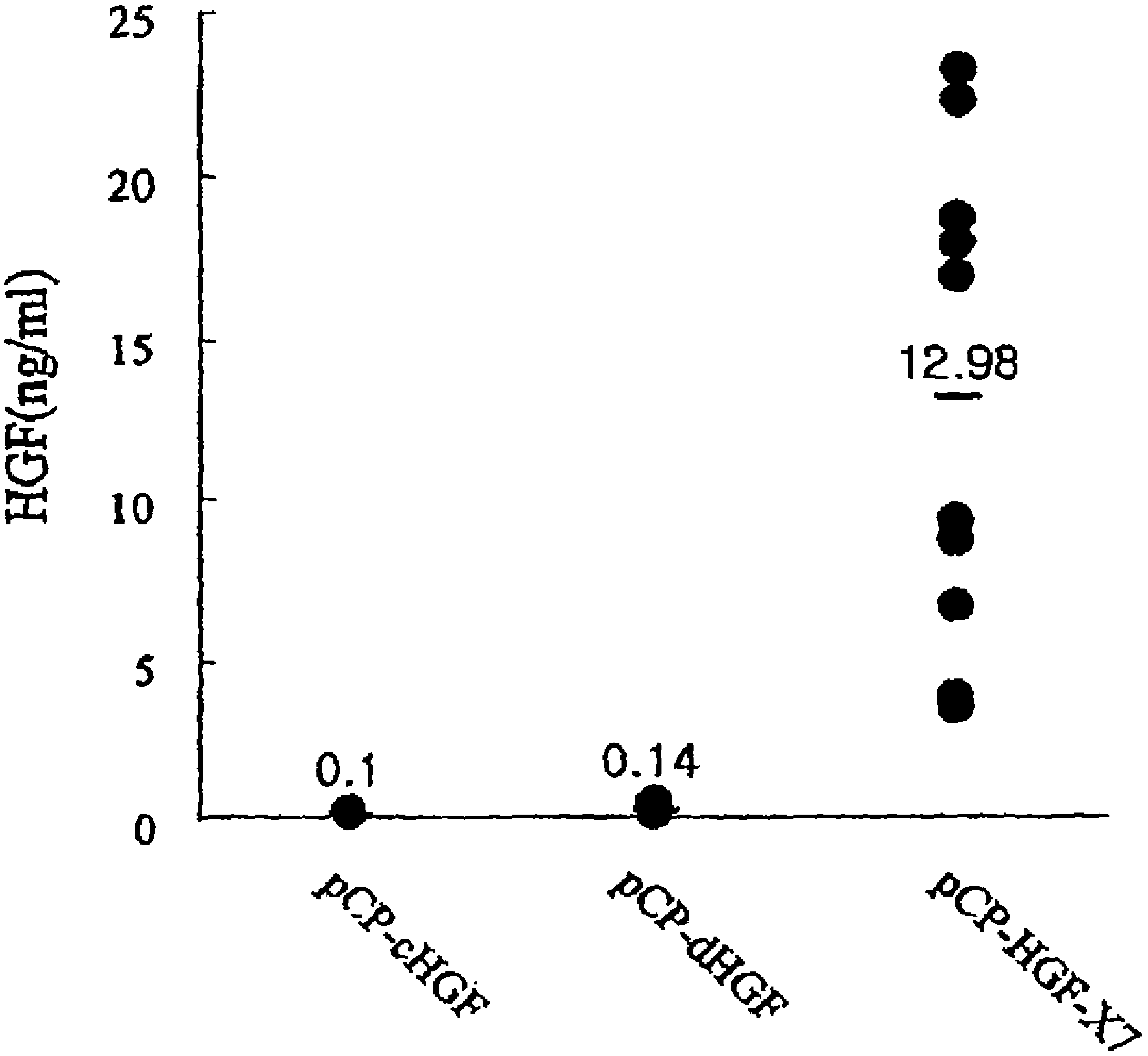
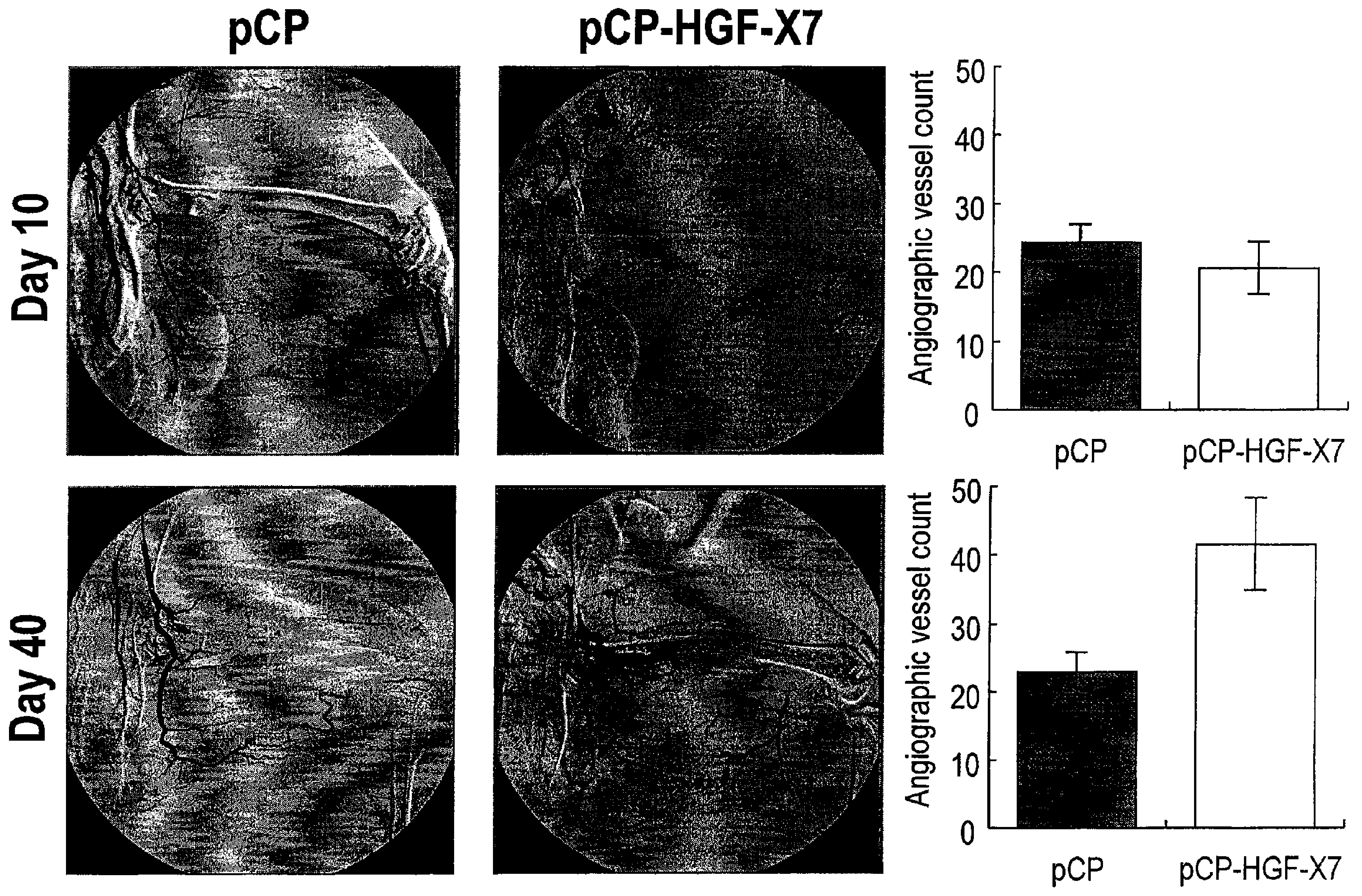


FIG. 11


pCP
pCP-HGF-X7
Day 10
Day 40
Angiographic vessel count
50
40
30
20
10
0
pCP
pCP-HGF-X7
Angiographic vessel count
50
40
30
20
10
0
pCP
pCP-HGF-X7

HYBRID HEPATOCYTE GROWTH FACTOR GENE HAVING HIGH EXPRESSION EFFICIENCY OF TWO HETEROTYPES OF HEPATOCYTE GROWTH FACTOR

This application is a continuation of U.S. application Ser. No. 10/944,277, filed Sep. 20, 2004, which is a continuation of International Application No. PCT/KR03/00548, filed Mar. 20, 2003, which claims priority benefit to Korean Appl. No. 10-2002-0015074, filed Mar. 20, 2002, each of which are herein incorporated by reference in their entirety.

REFERENCE TO A SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The content of the electronically submitted sequence listing ("sequencelisting.ascii.txt", 29,879 bytes, created on Dec. 30, 2009) filed with the application is incorporated herein by reference in its entirety.

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to a highly efficient hybrid Hepatocyte Growth Factor (HGF) gene which simultaneously expresses two heterotypes of HGF.

2. Related Art

The present invention relates to a hybrid HGF gene prepared by inserting an inherent or foreign intron between exons 4 and 5 in HGF cDNA, which has higher expression efficiency than HGF cDNA and simultaneously expresses two heterotypes of HGF and dHGF (deleted variant HGF).

HGF is a heparin binding glycoprotein called a scatter factor. A gene encoding HGF is located at chromosome 721.1 and comprises 18 exons and 17 introns, having the nucleotide sequence of SEQ ID NO: 1 (Seki T., et al., *Gene* 102:213-219 (1991)). A transcript of about 6 kb is transcribed from the HGF gene, and then, a polypeptide HGF precursor consisting of 728 amino acids is synthesized therefrom. Simultaneously, a polypeptide of dHGF precursor consisting of 723 amino acids is also synthesized by an alternative splicing of the HGF gene. The biologically inactive precursors may be converted into active forms of disulfide-linked heterodimer by protease in serum. In the heterodimers, the alpha chain having a high molecular weight forms four kringle domains and an N-terminal hairpin loop like a preactivated peptide region of plasminogen. The kringle domains of a triple disulfide-bonded loop structure consisting of about 80 amino acids may play an important role in protein-protein interaction. The low molecular weight beta chain forms an inactive serine protease-like domain. dHGF consisting 723 amino acids is a polypeptide with deletion of five amino acids in the 1st kringle domain of the alpha chain, i.e., F, L, P, S and S.

It has been recently reported that both of HGF and dHGF have several biological functions, e.g., promoting the growth and morphogenesis of epithelial cell, melanocyte and endothelial cell. However, they are different in terms of immunological or biological properties.

For example, HGF shows about 20-fold, 10-fold and 2-fold higher activities than dHGF in promoting DNA synthesis in human umbilical cord venous endothelial cell, arterial smooth muscle cell and NSF-60 (murine myeloblast cell), respectively. dHGF shows about 3-fold and 2-fold higher activities than HGF in promoting DNA synthesis of LLC-PK1 (pig kidney epithelial cell), and OK (American opossum kidney epithelial cell) and mouse interstitial cell, respectively. HGF has a 70-fold higher solubility in PBS than dHGF. Several anti-dHGF monoclonal antibodies recognize only dHGF, but not HGF or a reduced form of dHGF, which implies structures of HGF and dHGF are different. Accordingly, the simultaneous synthesis of HGF and dHGF in vivo suggests that they biologically interact with each other (Shima, N. et al., *Biochemical and Biophysical Research Communications* 200:808-815 (1994)).

HGF secreted from mesoderm-derived cells has various biological functions, e.g., 1) inducing epithelial cells into a tubular structure; 2) stimulating vascularization from endothelial cells in vitro and in vivo; 3) regeneration of liver and kidney, owing to its anti-apoptosis activity; 4) organogenesis of kidney, ovary and testis; 5) controlling osteogenesis; 6) stimulating the growth and differentiation of erythroid hematopoietic precursor cells; and 7) axon sprouting of neurons (Stella, M. C. and Comoglio, P. M., *The International Journal of Biochemistry & Cell Biology* 31:1357-1362 (1999)). Based on these various functions, HGF or a gene encoding HGF may be developed as a therapeutic agent for treating ischemic or liver diseases. Actually, in vivo, the HGF may exist as either HGF or dHGF, and therefore, the coexpression of HGF and dHGF is important for maximizing the therapeutic effect. Accordingly, the present inventors have endeavored to develop a hybrid HGF gene which can simultaneously express HGF and dHGF with a high efficiency for gene therapy.

SUMMARY OF THE INVENTION

Accordingly, it is a primary object of the present invention to provide a hybrid HGF gene which simultaneously expresses two heterotypes of HGF.

In accordance with one aspect of the present invention, there is provide the hybrid HGF gene having an inherent or foreign intron is inserted between exons 4 and 5 of HGF cDNA.

It is a another object of the present invention to provide a recombinant vector comprising the hybrid HGF gene and a microorganism transformed with the above vector.

It is a still further object of the present invention to provide a pharmaceutical composition for treating or preventing ischemic or liver diseases, which comprises the HGF gene.

BRIEF DESCRIPTION OF THE DRAWINGS

The above and other objects and features of the present invention will become apparent from the following description of the invention, when taken in conjunction with the accompanying drawings which respectively show:

FIG. **1**: a schematic diagram of HGF-X prototype illustrating the positions of the gene fragments,

FIG. **2**: a process for cloning gene fragments from HepG2 genomic DNA,

FIG. **3**: a process for cloning gene fragments from human placenta cDNA,

FIGS. **4**A and **4**B: processes for preparing expression vectors pCK-HGF-X,

FIG. **5**: a process for preparing expression vectors pCK-cHGF and pCK-dHGF,

FIG. **6**: a process for preparing expression vectors pCP-HGF-X family,

FIG. **7**: a process for preparing expression vectors pCP-cHGF and pCP-dHGF,

FIG. **8**: gene expression levels of pCP-cHGF, pCP-dHGF and pCP-HGF-X.

FIG. **9**: gene expression patterns of pCP-cHGF, pCP-dHGF and pCP-HGF-X observed by electrophoresis on 12% polyacrylamide gel,

FIG. **10**: gene expression levels of pCP-cHGF, pCP-dHGF and pCP-HGF-X7, in vivo,

FIG. **11**: cerebral angiogenesis of two groups of rabbits which were subject to administrating pCP and pCP-HGF-X7, respectively,

DETAILED DESCRIPTION OF THE INVENTION

The hybrid Hepatocyte Growth Factor (HGF) gene of the present invention comprises cDNA corresponding to the exons 1 to 18, and an inherent or foreign intron inserted between exons 4 and 5 of the cDNA. The intron comprises a fragment of the inherent intro or a recombinant sequence.

An embodiment of the hybrid HGF gene of the present invention comprising the inherent intron is 7113 bp long and has the nucleotide sequence of SEQ ID NO: 2. The hybrid HGF gene simultaneously expresses both HGF and dHGF, and has higher expression efficiency than HGF cDNA.

Codon degeneracy enables the hybrid HGF gene of the present invention to be modified or changed in the coding and/or non-coding region without altering the amino acid sequence of the protein and the expression of the gene. Accordingly, polynucleotides which is substantially identical to the hybrid HGF gene of SEQ ID NO:2, and the fragments thereof fall within the scope of the invention. “Substantially identical” means that the sequence homology is not less than 80%, preferably not less than 90%, and more preferably not less than 95%.

A hybrid HGF gene may comprise a fragment of inherent intron optionally having a small recombinant sequence inserted thereinto between exons 4 and 5 of HGF cDNA. Herein, such a hybrid HGF gene comprising a fragment of inherent intron designates “HGF-X”. HGFX-6, HGF-X7 and HGF-X8 having the nucleotide sequence of SEQ ID Nos: 19 to 21, respectively, are preferred.

The hybrid HGF gene of the present invention is synthesized and inserted into an expression vector, according to the known genetic engineering methods. Then, the vector can be introduced into an appropriate host cells such as *E. coli* and yeast. For example, *Escherichia coli* Top10F' may be transfected with HGF-X7 gene of the present invention. *Escherichia coli* Top10F' pCK-HGFX7 and *Escherichia coli* Top10F' pCP-HGFX7 then obtained were deposited as the accession numbers KCCM-10361 and KCCM-10362, respectively, on Mar. 12, 2002.

By using the transformed cells, the gene of the present invention and the protein encoded thereby may be produced on a large scale.

The vector of the present invention may selectively comprise sequence(s) for regulating gene expression such as promoter or terminator, self-replication sequence and secretory signal, depending on host cells.

Further, the present invention comprises a pharmaceutical composition for treating or preventing ischemic and liver diseases, which comprises the hybrid HGF gene or the vector comprising the gene as an active ingredient. Preferably, the composition is formulated for injection.

The composition of the present invention may further comprise pharmaceutically acceptable carriers. Any of the conventional procedures in the pharmaceutical field may be used to prepare oral formulations such as tablets, capsules, pills, granules, suspensions and solutions; rejection formulations such as solutions, suspensions, or dried powders that may be mixed with distilled water before injection; locally-applicable formulations such as ointments, creams and lotions; and other formulations.

Carriers generally used in the pharmaceutical field may be employed in the composition of the present invention. For example, orally-administered formulations may include binders, emulsifiers, disintegrating agents, excipients, solubilizing agents, dispersing agents, stabilizing agents, suspending agents, coloring agents or spicery. Injection formulations may comprise preservatives, unagonizing agents, solubilizing agents or stabilizing agents. Preparation for local administration may contain bases, excipients, lubricants or preservatives. Any of the suitable formulations known in the art (Remington's Pharmaceutical Science [the new edition], Mack Publishing Company, Eaton Pa.) may be used in the present invention.

The inventive composition can be clinically administered as various oral and parenteral formulations. A suitable formulation may be prepared using such excipients as additives, enhancers, binders, wetting agents, disintegrating agents and surfactants, or diluents. Solid formulations for oral administration include pills, tablets, dusting powder, granules and capsules. Those solid formulations may be prepared by mixing one or more excipients, e.g. starch, calcium carbonate, sucrose, lactose and gelatin with dibenzylbuthyllacton lignan derivatives. Also, lubricants such as magnesium stearate and talc may be included in the present formulation. Liquid formulations for oral administration include suspension, solution, emulsion and syrup. Those formulations may contain wetting agents, sweeteners, aromatics and preservatives, in addition to general simple diluents such as water and liquid paraffin. Formulations for parenteral administration include sterilized aqueous solution, suspension, emulsion, freeze-dried alternative treatment and suppositories. Water-insoluble excipients and suspending agents comprise vegetable fats such as propylene glycol, polyethylene glycol and olive oil, and injectable esters such as ethyl oleate. Witepsol®, Macrogol®, Tween® 61, cacao fats, laurin fats and glycerogelatins may be used as bases of suppositories.

The inventive composition may be administered orally or via parenteral routes such as intravenous, intramuscular, subcutaneous, intraabdominal, sternal and arterial injection or infusion, or topically through rectal, intranasal, inhalational or intraocular administration.

It should be understood that the typical daily dose of composition of the present invention ought to be determined in light of various relevant factors including the conditions to be treated, the chosen route of administration, the age, sex and body weight of the individual patient, and the severity of the patient's symptom, and can be administrated in a single dose or in divided dose. Therefore, the daily dose should not be construed as a limitation to the scope of the invention in any way.

The following Examples are given for the purpose of illustration only, and are not intended to limit the scope of the invention.

EXAMPLE 1

Preparation of Hybrid Gene Constructs Encoding Human HGF

(1) Cloning of HGF Gene Fragments Obtained from Genomic DNA

Human HepG2 cells (ATCC Accession NO: HB-8065) were suspended in TES buffer (10 mM Tris-HCl; 1 mM

EDTA; 0.7% SDS) and treated with 400 μg/Ml of proteinase K at 50° C. for 1 hour. Subsequently, genomic DNA was extracted from the cell suspension by phenol/chloroform extraction and ethanol precipitation according to the conventional method in the art.

In the PCR amplification, the extracted genomic DNA was employed as a template DNA. As primer pairs, the synthetic nucleotides of SEQ ID NOs: 3 and 4 were employed to obtain DNA fragments containing: HGF gene fragment 2 (HGF-F2), SEQ ID NOs: 3 and 5; HGF-F3, SEQ ID NOs: 6 and 7; HGF-F5, SEQ ID NOs: 8 and 7; HGF-F7, SEQ ID NOs: 9 and 7; HGF-F8, SEQ ID NOs: 10 and 7; HGF-F6, respectively (FIG. **1**). The PCR amplification mixture was prepared by mixing 1 μl of template DNA, 1 μl each of primer (10 pmol/μl), 10 μl of dNTP (10 mM), 3.5 unit of Expand High Fidelity enzyme (Gibco BRL, USA) and 10 μl of enzyme buffer solution and adjusted to a final volume of 100 μl with distilled water. 30 cycles of the PCR amplification was carried out, each cycle consisting of 1 min at 94° C., 1 min at 55° C. and 30 sec at 72° C. The primers used herein and the amplified gene fragments obtained therefrom are shown in Table 1.

TABLE 1

5' primer	3' primer	Amplified fragment
gHGF3 (SEQ ID NO: 3)	gHGF4 (SEQ ID NO: 4)	HGF gene fragment 2 (HGF-F2)
gHGF3 (SEQ ID NO: 3)	gHGF10 (SEQ ID NO: 5)	HGF gene fragment 3 (HGF-F3)
gHGF5 (SEQ ID NO: 6)	gHGF7 (SEQ ID NO: 7)	HGF gene fragment 5 (HGF-F5)
gHGF12 (SEQ ID NO: 8)	gHGF7 (SEQ ID NO: 7)	HGF gene fragment 7 (HGF-F7)
gHGF13 (SEQ ID NO: 9)	gHGF7 (SEQ ID NO: 7)	HGF gene fragment 8 (HGF-F8)
gHGF6 (SEQ ID NO: 10)	gHGF7 (SEQ ID NO: 7)	HGF gene fragment 6 (HGF-F6)

The amplified HGF-F2 comprised the sequence ranging from 392 to 2247 of human HGF cDNA prototype (HGF-X1; composed of exons 1 to 4-intron 4-exons 5 to 18) of SEQ ID NO: 2; HGF-F3, the sequence ranging from 392 to 727; HGF-5, the sequence ranging from 2229 to 5471; HGF-F6, the sequence ranging from 5117 to 5471; HGF-F7, the sequence ranging from 3168 to 5471; and HGF-F8, the sequence ranging from 4168 to 5471.

The amplified HGF gene fragments were each inserted into the multiple cloning site of pGEM-T easy vector (Promega, WI, USA) to obtain pGEM-T easy-HGF-F2, pGEM-T easy-HGF-F3, pGEM-T easy-HGF-F5, pGEM-T easy-HGF-F6, pGEM-T easy-HGF-F7 and pGEM-T easy-HGF-F8, respectively (FIG. **2**). The nucleotide sequences of the amplified HGF gene fragments were confirmed by a sequence analysis.

(2) Cloning of HGF Gene Fragments Obtained from cDNA

In the PCR amplification, human placenta cDNA (Clontech, CA, USA) was employed as a template DNA under the same condition as described in Example 1. As primer pairs, the synthetic oligonucleotides of SEQ ID NOs: 11 and 12, and SEQ ID NOs: 13 and 14 were employed to obtain DNA fragments containing HGF-F1 and HGF-F4, respectively. Further, DNA fragments containing cDNAs of HGF gene (cHGF) and deleted HGF gene (dHGF) were amplified by PCR using synthetic oligonucleotides of SEQ ID NOs: 15 and 16 as a primer pair, respectively. dHGF is a HGF gene with deletion of 5 base sequences.

The primers used herein and the amplified gene fragments obtained therefrom are shown in Table 2.

TABLE 2

5' primer	3' primer	Amplified fragment
gHGF1 (SEQ ID NO: 11)	gHGF2 (SEQ ID NO: 12)	HGF gene fragment 1 (HGF-F1)
gHGF8 (SEQ ID NO: 13)	gHGF9 (SEQ ID NO: 14)	HGF gene fragment 4 (HGF-F4)
cHGF5 (SEQ ID NO: 15)	cHGF3 (SEQ ID NO: 16)	HGF gene cDNA (cHGF) dHGF gene cDNA (dHGF)

The amplified HGF-F1 and HGF-F4 comprised the nucleotide sequences ranging from 1 to 402 and from 6533 to 7113 of SEQ ID NO: 2 of human HGF cDNA prototype, respectively. HGF gene cDNA comprised the nucleotide sequence ranging from 1 to 2184 of SEQ ID NO: 1 of human HGF gene, and dHGF gene cDNA has the same sequence as HGF gene cDNA except for the deletion of the sequence ranging from 483 to 495.

The amplified fragments of HGF gene were each inserted into the multiple cloning site of pGEM-T easy vector (Promega, WI, USA) to obtain pGEM-T easy-HGF-F1, pGEM-T easy-HGF-F4, pGEM-T easy-cHGF and pGEM-T easy-dHGF, respectively (FIG. **3**). The nucleotide sequences of the human HGF gene fragments, HGF gene cDNA and dHGF gene cDNA were confirmed by sequence analyses.

(3) Preparation of Hybrid HGF Gene Constructs

Hybrid HGF gene constructs of genomic DNA and cDNA were prepared by combining the fragments of HGF gene cloned in steps (1) and (2) as follows (FIGS. **4**A and **4**B).

Plasmid pGEM-T-easy-HGF-F1 was treated with HindIII/BamHI to obtain HGF-F1. Plasmid pCK (see PCT International Publication NO: WO/0040737) was treated with HindIII/BamHI, and HGF-F1 was inserted thereinto to obtain pCK-F1. And then, plasmids pGEM-T-easy-HGF-F2 and pGEM-T-easy-HGF-F3 were treated with MluI/BamHI to obtain HGF-F2 and HGF-F3, respectively. pCK-1 was treated with MluI/BamHI, and then HGF-F2 and HGF-F3 were inserted thereinto to obtain pCK-F12M and pCKF13M. The MluI restriction site of vectors pCK-F12M and pCK-F13M was substituted with an HgaI restriction site by employing a site-directed mutagenesis kit (Stratagene, CA., USA) to obtain pCK-F12 and pCK-F13, respectively.

Further, plasmid pGEM-T-easy-HGF-F4 was treated with BamHI/XbaI to obtain HGF-F4. pCK-F12 and pCK-F13 were treated with BamHI/XbaI, and HGF-F4 was inserted thereinto to obtain pCK-F124 and pCK-F134, respectively. And then, plasmids pGEM-T-easy-HGF-F5, pGEM-T-easy-HGF-F6, pGEM-T-easy-HGF-F7 and pGEM-T-easy-HGF-F8 were treated with BamHI/XhoI to obtain HGF-F5, HGF-F6, HGF-F7 and HGF-F8, respectively. pCK-F124 and pCK-F134 were treated with BamHI/XhoI, and then HGF-F5, HGF-F6, HGF-F7 and HGF-F8 were inserted thereinto to obtain pCK-F1254 and pCK-F1264, pCK-F1274, pCK-F1284, pCK-F1354, pCK-F1364, pCK-F1374 and pCK-F1384, respectively.

And then, pGEM-T easy-cHGF was treated with XhoI to obtain cDNA fragment of HGF gene (HGF-XhoI) of about 1100 bp. Then, HGF-XhoI was inserted into pCK-F1254, pCK-F1264, pCK-F1274, pCK-F1284, pCK-F1354, pCK-F1364, pCK-F1374 and pCK-F1384 to obtain pCK-HGF-X1, pCK-HGF-X2, pCK-HGF-X3, pCK-HGF-X4, pCK-HGF-X5, pCK-HGF-X6, pCK-HGF-X7 and pCK-HGF-X8, respectively. On the other hand, pGEM-T easy-cHGF and pGEM-T easy-dHGF were treated with BamHI to obtain HGF gene cDNA and dHGF gene cDNA. Then, HGF gene cDNA and dHGF gene cDNA were inserted into the BamHI restriction site of pCK to obtain pCK-cHGF and pCK-dHGF, respectively (FIG. **5**).

(4) Preparation of an Expression Vector Containing a Hybrid HGF Gene Construct

Plasmid pcDNA3.1 (Invitrogen, USA) was digested with NdeI, treated with the Klenow fragment to build blunt ends, and then digested with NheI to obtain a DNA fragment containing human cytomegalovirus promoter. Plasmids pCK-HGF-X1, pCK-HGF-X2, pCK-HGF-X3, pCK-HGF-X4, pCK-HGF-X5, pCK-HGF-X6, pCK-HGF-X7 and pCK-HGF-X8 were digested with SnaBI, treated with the Klenow fragment to make blunt ends and digested with NheI, and then the above DNA fragment containing human cytomegalovirus promoter was inserted thereinto to obtain pCP-HGF-X1, pCP-HGF-X2, pCP-HGF-X3, pCP-HGF-X4, pCP-HGF-X5, pCP-HGF-X6, pCP-HGF-X7 and pCP-HGF-X8, respectively (FIG. **6**).

Plasmid pcDNA3.1 (Invitrogen, USA) was digested with NheI, treated with the Klenow fragment to make blunt ends and digested with NdeI to obtain the DNA fragment containing human cytomegalovirus promoter. pCK-cHGF and pCK-dHGF were digested with MluI, treated with the Klenow fragment to make blunt ends and digested with NdeI, and then the above DNA fragment containing human cytomegalovirus promoter was inserted thereinto to obtain pCP-cHGF and pCP-dHGF, respectively (FIG. **7**).

EXAMPLE 2

Examination of the Expression Efficiency of Hybrid HGF Gene Construct and the Coexpression of HGF/dHGF

Studies was conducted to examine whether the hybrid HGF gene constructs (HGF-X1 to HGF-X8) obtained in Example 1 can simultaneously express HGF and dHGF and whether there is any difference in the gene expression level between hybrid HGF gene constructs and HGF cDNA.

(1) Gene Expression Efficiency

First, 5 μg of pCP-HGF-X2, pCP-HGF-X3, pCP-HGF-X6, pCP-HGF-X7 and pCP-HGF-X8 were transfected into 5×10^6 cells of 293 cell (ATCC CRL 1573) together with 0.5 μg of DONAI-LacZ (TAKARA SHUZO, Japan) DNA using FuGENE6 (Gibco BRL, MD, USA), according to the manufacturer's instructions. At this time, 5 μg each of pCP-cHGF and pCP-dHGF were used as controls, and DONAI-LacZ DNA was used to calibrate the infection efficiency. 3 hours after transfection, cells were re-fed with a fresh medium and further cultured for 48 hours. The culture solution thus obtained was divided into two parts. One part of the 293 cell culture solution was subjected to RNA extraction, and the other, to measurement of LacZ activity. The LacZ activity was measured using an activity measuring kit (Stratagene, CA, USA) according to the manufacturer's instructions.

In order to compare the gene expression levels, the amount of HGF in the cell culture was measured by an enzyme-linked immunosorbent assay kit (ELISA, R&D System, MN, USA). After calibrating the infection efficiency by the measured LacZ activity, the expression level of HGF-X gene was found to be from 20 to 150-fold higher than those of HGF cDNA and dHGF cDNA (FIG. **8**). HGF-X7, in particular, showed the highest gene expression level.

(2) Coexpression of HGF and dHGF

In order to examine coexpression of HGF and dHGF from hybrid HGF gene constructs, total cellular RNAs were extracted from the transfected 293 cells using the Trizol method (Trizol; Gibco BRL, USA) and subjected to RT-PCR to obtain cDNA. Then, using cDNA as a template DNA, PCR amplification was carried out using synthetic oligonucleotides of SEQ ID NOs: 17 and 18 as a primer pair. The PCR amplification mixture was prepared by mixing 1 μl of the template DNA, 1 μl each of the primer (10 pmol/μl), 10 μl of dNTP (10 mM), 3.5 unit of Taq polymerase (TAKARA SHUZO, Japan) and 10 μl of enzyme buffer solution and adjusted to a final volume of 100 μl with distilled water. 30 cycles of PCR amplification was conducted, each cycle consisting of 1 min at 94° C., 1 min at 55° C., and 90 sec at 72° C.

The amplified PCR products corresponded to the boundary region between exons 4 and 5 of HGF gene; HGF gene cDNA of 142 by and dHGF gene cDNA of 127 bp, respectively. With no splicing, the PCR product of at least 1 kb in length was amplified; and if alternative splicing occurred, HGF gene cDNA of 142 bp and dHGF gene cDNA of 127 by were simultaneously synthesized and amplified. The amplified PCR products were distinguished by electrophoresis on a 12% polyacrylamide gel.

As shown in FIG. **9**, while the bands of 142 by and 127 by were detected in the lanes loading HGF gene cDNA and dHGF gene cDNA, respectively, both bands of 142 by and 127 by were detected in the lanes loading HGF-X. The above results suggest that HGF and dHGF are simultaneously expressed from hybrid HGF-X gene constructs of the present invention.

EXAMPLE 3

Comparison of Expression Levels of HGF-X7, HGF Gene cDNA and dHGF Gene cDNA (In Vivo)

100 μg each of pCP-HGF-X7, pCP-cHGF and pCP-dHGF were injected into the enterior tibial muscle of the hind limb of mice with an insulin syringe. After 5 days, the mice were sacrificed and the muscles around the injection spot were removed and smashed in a protein extraction buffer (25 mM Tris-HCl (pH 7.4), 50 mM NaCl, 0.5% Na-deoxycholate, 2% NP-40, 0.2% SDS) to separate total proteins. The amount of the total proteins was measured with a DC protein analysis kit (Bio-Rad Laboratories, CA, USA) and the amount of expressed HGF was determined with an ELISA kit (R&D System) according to the manufacturer's instruction.

As can be seen from the result shown in FIG. **10**, the amount of HGF expressed from HGF-X7 is 250-fold higher than that from HGF gene cDNA or dHGF gene cDNA.

Together with the result of the experiment of Example 2 (in vivo), this result demonstrates that the expression efficiency of HGF-X gene is much superior to those of HGF gene cDNA or dHGF gene cDNA.

EXAMPLE 4

Gene Therapy Employing HGF-X7 in a Rabbit Ischemic Hind Limb Model

In order to examine whether HGF-X7 gene is effective in the treatment of ischemic hind limb disease, gene therapy was carried out on a rabbit ischemic hind limb model as follows.

A rabbit ischemic hind limb model, which is a standard animal model for the ischemic limb disease, was prepared by the method described by Takeshita et al., *Journal of Clinical*

Investigation 93:662 (1994). At the day before operation (day 0), each of 30 white rabbits from New Zealand (male, from 3.8 to 4.2 kg) was intramuscularly injected with 5 mg/kg of xylazine and, then, anesthetized by an intramuscular injection of 50 mg/kg of ketamine. Subsequently, the left femoral region of the rabbit was incised and all branches of the femoral artery were separated and tied. The region from the proximal part to the branching point of the saphenous and popliteal arteries was incised to prepare the model. After the operation, 15 mg/kg/day of cefazolin was injected intramuscularly for 5 days and 0.3 mg/day of morphine, for 10 days. 10 days after the operation (day 10), angiography was carried out for the left hind limb where the ischemia was induced, and the degree of arteriogenesis was recorded as a basal level. The rabbits were randomly divided into two groups and injected at four sites in the femoral muscle with 500 μg of plasmid pCP-HGF-X7 (experimental group) or 500 μg of plasmid pCP (control), respectively. 40 days after the operation (day 40), angiography was carried out again for the left hind limb and the degree of arteriogenesis at the arteriole level was determined and compared to that of day 10.

As can be seen from the result in FIG. **11**, the degree of angiogenesis was significantly enhanced in the experimental group administered with pCP-HGF-X7 as compared with the pCP-administered control group.

This result demonstrates that HGF-X7 gene can be effectively used in the gene therapy of an ischemic disease.

While the invention has been described with respect to the above specific embodiments, it should be recognized that various modifications and changes may be made to the invention by those skilled in the art which also fall within the scope of the invention as defined by the appended claims.

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<210> SEQ ID NO 3
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF3 primer

<400> SEQUENCE: 3

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<210> SEQ ID NO 4
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF4 primer

<400> SEQUENCE: 4

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<210> SEQ ID NO 5
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF10 primer

<400> SEQUENCE: 5

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<210> SEQ ID NO 6
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:

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<223> OTHER INFORMATION: gHGF5 primer

<400> SEQUENCE: 6

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<210> SEQ ID NO 7
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF7 primer

<400> SEQUENCE: 7

cccctcgagg atttcgacag tagtttt                                         27


<210> SEQ ID NO 8
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF12 primer

<400> SEQUENCE: 8

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<210> SEQ ID NO 9
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF13 primer

<400> SEQUENCE: 9

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<210> SEQ ID NO 10
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF6 primer

<400> SEQUENCE: 10

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<210> SEQ ID NO 11
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF1 primer

<400> SEQUENCE: 11

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<210> SEQ ID NO 12
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
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<223> OTHER INFORMATION: gHGF2 primer

<400> SEQUENCE: 12

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<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF8 primer

<400> SEQUENCE: 13

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<210> SEQ ID NO 14
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: gHGF9 primer

<400> SEQUENCE: 14

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<210> SEQ ID NO 15
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: cHGF5 primer

<400> SEQUENCE: 15

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<210> SEQ ID NO 16
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: cHGF3 primer

<400> SEQUENCE: 16

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<210> SEQ ID NO 17
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GHGF5' primer

<400> SEQUENCE: 17

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<210> SEQ ID NO 18
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: GHGF3' primer

<400> SEQUENCE: 18

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<210> SEQ ID NO 19
<211> LENGTH: 4679
<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HGF-X6 gene
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accaaaaaag tgaatactgc agaccaatgt gctaatagat gtactaggaa taaaggactt    240

ccattcactt gcaaggcttt tgtttttgat aaagcaagaa aacaatgcct ctggttcccc    300

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aacaaagact acattagaaa ctgcatcatc ggtaaaggac gcagctacaa gggaacagta    420

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aggtaagaac agtatgaaga aaagagatga agcctctgtc ttttttacat gttaacagtc    540

tcatattagt ccttcagaat aattctacaa tcctaaaata acttagccaa cttgctgaat    600

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<210> SEQ ID NO 20
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<212> TYPE: DNA
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: HGF-X7 gene

<400> SEQUENCE: 20

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gaattcaaaa aatcagcaaa gactacccta atcaaaatag atccagcact gaagataaaa 180

accaaaaaag tgaatactgc agaccaatgt gctaatagat gtactaggaa taaaggactt 240

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aacaaagact acattagaaa ctgcatcatc ggtaaaggac gcagctacaa gggaacagta 420

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What is claimed is:

1. A method for co-expressing two heterotypes of Hepatocyte Growth Factor (HGF) comprising transforming or transfecting to a cell a DNA construct comprising:

(a) a promoter,

(b) a first cDNA which has the same sequence as exons 1-4 of the human HGF gene wherein said exons 1-4 are arranged in sequential order without an intron therebetween, or degenerates thereof which do not alter the amino acid sequence encoded by said first cDNA,

(c) a polynucleotide that has the same sequence as intron 4 of the HGF gene or a functional fragment thereof, and

(d) a second cDNA which has the same sequence as exons 5-18 of the human HGF gene wherein said exons 5-18 are arranged in sequential order without an intron therebetween, or degenerates thereof which do not alter the amino acid sequence encoded by said second cDNA;

wherein (c) is located between (b) and (d); and the HGF construct simultaneously encodes two heterotypes of human HGF.

2. The method of claim **1**, wherein said intron has the same sequence as a fragment of intron 4 of the HGF gene.

3. The method of claim **2**, wherein the construct comprises a nucleotide sequence not less than 90% identical to SEQ ID NO: 19.

4. The method of claim **3**, wherein the construct comprises a nucleotide sequence not less than 95% identical to SEQ ID NO: 19.

5. The method of claim **4**, wherein the construct comprises the sequence of SEQ ID NO: 19.

6. The method of claim **2**, wherein the construct comprises a nucleotide sequence not less than 90% identical to SEQ ID NO: 20.

7. The method of claim **6**, wherein the construct comprises a nucleotide sequence not less than 95% identical to SEQ ID NO: 20.

8. The method of claim **7**, wherein the construct comprises the sequence of SEQ ID NO: 20.

9. The method of claim **2**, wherein the construct comprises a nucleotide sequence not less than 90% identical to SEQ ID NO: 21.

10. The method of claim **9**, wherein the construct comprises a nucleotide sequence not less than 95% identical to SEQ ID NO: 21.

11. The method of claim **10**, wherein the construct comprises the sequence of SEQ ID NO: 21.

12. The method of claim **1**, wherein the one intron has the same sequence as the full intron 4 of the HGF gene.

13. The method of claim **1**, wherein the construct comprises a nucleotide sequence not less than 90% identical to SEQ ID NO: 2.

14. The method of claim **13**, wherein the construct comprises a nucleotide sequence not less than 95% identical to SEQ ID NO: 2.

15. The method of claim **14**, wherein the construct comprises the sequence of SEQ ID NO: 2.

16. The method of claim **1**, wherein the construct further comprises a terminator sequence, a self-replication sequence, or a secretory signal.

17. The method of claim **1**, wherein the expression efficiency of the construct is higher than the expression efficiency of HGF cDNA or deleted variant HGF (dHGF) cDNA.

18. The method of claim **1**, wherein the expression level of the construct is about 20- to 100-fold higher than the expression level of the HGF cDNA or dHGF cDNA.

19. The method of claim **1**, wherein the cell is a mammalian cell, a bacterial cell or a yeast cell.

20. The method of claim **19**, wherein the cell is a mammalian cell.

21. The method of claim **20**, wherein the transformation of said mammalian cell is in vivo.

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